INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP01/02806

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7] C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[7] C12Q1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
BIOSIS (DILOG)
WPI (DIALOG)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X/Y	WO, 93/21340, A (MEDICAL RES COUNCIL), 28 October, 1993 (28.10.93), Claims 1,2; page 19, line 32 to page 20, line 4; page 21, line 24 to page 22, line 11 & EP, 640146, A & JP, 7-507681, A & US, 6087095, A	1-5,7,8/6,9
Y	WO, 90/13666, A (AMERSHAM INT PLC), 15 November, 1990 (15.11.90) & EP, 471732, A & JP, 4-505251, A	1-9
Y	WO, 91/06678, A (SRI INT), 16 May, 1991 (16.05.91) & EP, 450060, A & JP, 4-503460, A	1-9
Y	WO, 94/23064, A (INST PASTEUR), 13 October, 1994 (13.10.94) & EP, 690928, A & JP, 8-508473, A & US, 5798210, A	1-9
Y	WO, 93/05183, A (BAYLOR COLLEGE OF MEDICINE), 18 March, 1993 (18.03.93)	1-9

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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Date of the actual completion of the international search	Date of mailing of the international search report
23 April, 2001 (23.04.01)	15 May, 2001 (15.05.01)
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	& AU, 9226740, A	
Y	Yoshihiro TAKEDA, et al., "Kyou Shouten Laser Keikou Kenbikyou ni yoru Youekichuu no Tanitsu Shikiso Bunshi no Kenshutsu", Bunko Kenkyu, Vol.49, No.1 (February, 2000) pp.17-18	6
Y	WO, 98/58240, A (Toyota Motor Corporation), 23 December, 1998 (23.12.98) & JP, 11-502041, A	9

分光便利帳

共焦点レーザー蛍光顕微鏡による溶液中の単一色素分子の検出

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溶液中の溶質分子と周囲の溶媒分子との相互作用やそのゆらぎは，多数の溶質分子を集団として観測するだけでは捕らえにくく，個々の溶質分子を検出，同定することが必要である．個々の分子を溶液中で検出するにはいくつかの方法があるが，本稿では共焦点レーザー蛍光顕微鏡による溶液中の単一色素分子の検出について述べる．

共焦点の光学系を組み込んだレーザー蛍光顕微鏡では，微小な観測領域からの蛍光信号だけを取り出すことができる．これは，対物レンズによりレーザー励起光が直径約0.8 μm の焦点領域にまで絞られるということと，共焦点の光学系により，観測領域が深さ方向に約1 μm に制限されるということによっている．さらに，試料溶液中の色素分子の濃度を10^{-10} M 以下にすると，ある時間には観測領域に一個の分子しか存在せず，単一の色素分子からの蛍光信号のみを検出できる．色素分子は励起光を吸収して寿命約10 ns 程度で蛍光を放出し基底状態にもどるが，十分な強度の連続発振のレーザー励起光の照射下では，この蛍光分子はただちに励起光で励起されるので，励起光の吸収と蛍光の放出を約10 ns 程度で繰り返し，毎秒10^8 個もの蛍光光子を放出する．これを量子収率70％のアバランシェフォトダイオードで検出すると，装置全体で検出効率は1％程度になり，単一の色素分子を検出することができる．

図1に示すように，実験装置は光源のアルゴンイオンレーザー（Spectra-Physics 社 BeamLok 2060-7S），倒立型顕微鏡（オリンパス IX70），及び検出部からなる．ダイクロイックミラー（オリンパス DM505）で反射させたアルゴンイオンレーザー励起光を油浸対物レンズ（オリンパス UplanApo 100×Oil I）を用いて色素溶液中に絞り込み，照射する．色素分子からの蛍光信号は同じ対物レンズを戻り，バンドパスフィルターを通して取り出される．この蛍光信号をアバランシェフォトダイオード（EG & G 社 SPCM-AQ）を用いて検出し，マルチチャネルカウンター（Stanford Research Systems 社 SR430）で光子計数を行う．また，励起微小領域（5×10^{-16} l）からの蛍光信号のみを取り出すために油浸対物レンズの焦点と共役の位置に直径40 μm のピンホールを置き，共焦点光学系を形成する．

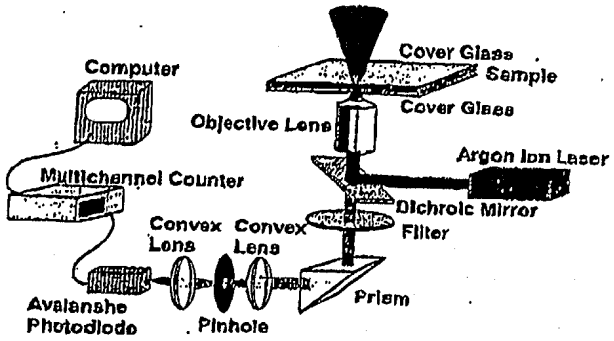

図1　共焦点レーザー蛍光顕微鏡の装置図．

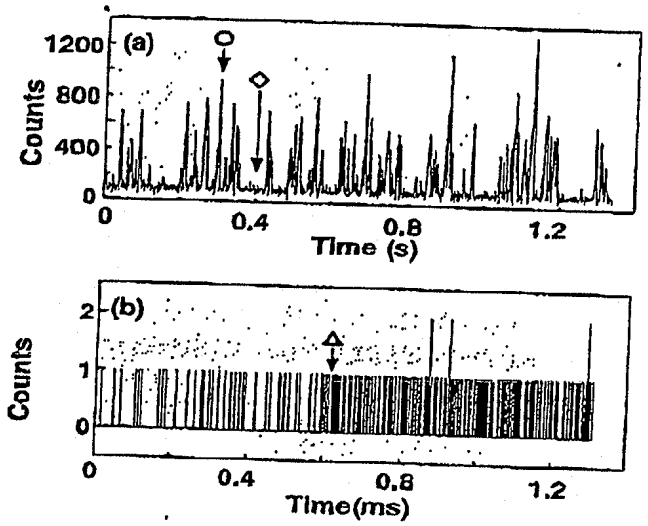

図2　10^{-10} M のローダミン6G の水とエタノール1：1溶液の蛍光信号の時間依存性．(a)マルチチャンネルカウンターのゲート時間を1.34 ms に，時間軸のデータポイント数を1 k に設定した．○はバンチング，◇はアンチバンチング領域を示す．(b)マルチチャンネルカウンターのゲート時間を80 ns に，時間軸のデータポイント数を16 k に設定した．△は一個の色素分子が観測領域に入ってきた時間を示す．

まず10^{-2} M 程度の濃い色素溶液を蛍光顕微鏡のカバーガラス上に置く．レーザー励起光がこの試料の中に焦点を結ぶように油浸対物レンズの高さを微調整する．この試料からの蛍光を肉眼で見ながら，マイクロメーターを用いてピンホールやレンズなどの光学系の位置合わせを行う．その後，この試料を測定用の希薄な試料に置き変えて測定を続行する．但し，アバランシェフォトダイオードは，その受光面の直径（0.2 mm）が小さく，正確な位置合わせを肉眼でおこなうのは不可能である．そのため，大体の位置合わせを濃厚溶液で行った後，測定用の希薄溶液に置き換え，レーザー励起光を照射し，単位時間当たりの蛍光光子のカウント数が最大になるようにマイクロメーターでアバランシェフォトダイオードの位置の微調整を行う．

一方，試料の調整のためには，ガラス表面を洗浄する必要がある．特に，色素分子を水に溶かした場合，洗浄が充分でないと，ガラス表面に色素が吸着する．我々は，ガラス表面をアセトンで脱脂した後，1 M KOH 処理し，30% H_2O_2 と濃 H_2SO_4 の2：1溶液で処理をしている．このような処理により，ガラス表面の親油性の成分が除去され，OH 基が表面に再生され，親水性になる．

溶媒として水，アルコール類を使用した場合は，OH 伸縮振動によるラマン散乱が大きな背景雑音となる．例えば，488 nm の励起光でローダミン6Gの蛍光（最大蛍光波長556 nm）を測定した場合，このラマン散乱は575 nm 付近に現われ，蛍光信号との重なりが大きくなる．迷光やラマン散乱光に対して充分に強い蛍光を観測するためには，励起光の波長や強度の調整や適切なバンドパスフィルターの選択が必要である．励起光の波長に関しては，色素分子の最大吸収波長（530 nm）に励起光の波長を合わせると，励起波長と蛍光波長が近いため，蛍光信号と迷光の分離が難しくなる．一方，励起波長をこれより短くすると，蛍光信号と迷光の分離は良くなるが，この励起波長での色素の吸収係数が低くなるため蛍光信号が弱くなることと，ラマン散乱が蛍光シグナルに重なってくるという難点がある．励起光の強度に関しても，最適値があると考えられる．強度の増加に従って迷光とラマン散乱が大きくなり，さらに蛍光色素の消光が起こるようになる．一方，強度が小さいと色素分子が基底状態に戻った後，再び励起されるまで時間を要するようになるため，蛍光信号が弱くなる．今回，図2に例示しているような系では，励起波長が514 nm，強度が0.5 mW にすると最も良い結果が得られる．またバンドパスフィルターに関しても，最適な透過波長とその半値幅を決めておく必要がある．ラマン散乱や迷光などの背景雑音を最小にし，信号強度をあまり落とさないようにするよう考慮し，オメガオプティクス社のバンドパスフィルター BP545-580（帯域幅545 nm-580 nm）を用いている．

図2aにマルチチャンネルカウンターのゲート時間を1.34 ms に設定した場合の蛍光の時間依存性を示す．ローダミン6G溶液の濃度が10^{-10} M 以下で単一分子検出が可能になる．すなわち，観測領域にブラウン運動で入ってきたローダミン6G分子が励起光の吸収と蛍光の放出を寿命約10 ns 程度で繰り返し，約10^8個/s の光子を放出することが観測される（バンチング）．さらにこの分子が観測領域から出て行くと蛍光信号は消失する（アンチバンチング）．また，ローダミン6Gの観測領域内のブラウン運動による滞在時間は約1 ms 程度と考えられるので，この間に一個の色素分子が放出する光子数は10^5個程度になる．測定される光子数が1000個程度であるので，検出系は1％程度の効率で光子を検出していることになる．また図2bにマルチチャンネルカウンターのゲート時間を80 ns に変えた結果を示す．単一の色素分子は約10 ns 程度の平均周期で励起光吸収と蛍光放出をランダムに繰り返している様子がわかる．

DETECTION OF SINGLE DYE MOLECULES IN SOLUTION USING CONFOCAL LASER FLUORESCENCE MICROSCOPE

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It is difficult to capture the interaction or fluctuation between a solute molecule and a surrounding solvent molecule in a solution only by observing many solute molecules as a group. To do this, detection and identification of individual solute molecules are required. There exist several techniques to detect individual molecules in a solution. In this article we will describe the detection of single dye molecules in a solution using a confocal laser fluorescence microscope.

A laser fluorescence microscope with confocal optical system incorporated therein can collect fluorescent signals only from a microarea for observation. This is because laser-excited light is focused by objective lens so that focus area comes at a diameter of approximately 0.8μm, and because the observation area is limited to approximately 1μm in depth direction by the confocal optical system. Further, when the concentration of dye molecules in a sample solution is 10^{-10} M or less, only fluorescent signals from a single dye molecule can be detected because only one molecule is present in the observation area in certain time. Dye molecule absorbs the excited light, emits fluorescence with its life span of approximately 10ns, and returns to its ground state. Under irradiation of laser-excited light which continuously oscillates with a sufficient intensity, fluorescent molecule is immediately excited by the excited light, so that the absorption of excited light and the emission of fluorescence are repeated at intervals of approximately 10ns, releasing as much as 10^8 fluorescent photons per second. When the photons are detected by Avalanshe photodiode in a quantum yield of 70%, the detection efficiency becomes around 1% in the whole system,

by which single dye molecules can be detected.

As shown in Fig. 1, the experimental system comprises an argon ion laser as a light source (Spectra-Physics, BeamLok 2060-7S), an inverted microscope (Olympus IX70) and a detector. Argon ion laser-excited light reflected from a dichroic mirror (Olympus DM505) is focused and irradiated into a dye solution using an oil-immersed objective lens (Olympus UplanApo 100xOil I). Fluorescent signals from dye molecules return to the same objective lens, and are collected through a band-pass filter. The fluorescent signal is detected using an Avalanshe photodiode (EG & G, SPCM-AQ), and then the number of photons is counted using a multi channel counter (Stanford Research Systems, SR430). In addition, to collect only the fluorescent signals from excited microarea ($5x10^{-10}l$), a pinhole with a diameter of 40μm is placed at a position conjugating with the focal point of the oil-immersed objective lens, thereby forming a confocal optical system.

First, a concentrated dye solution is placed at about $10^{-2}M$ on a cover glass of the fluorescent microscope. The height of an oil-immersed objective lens is finely adjusted for laser-excited light to focus into the sample. An optical system including a pin hole and lens is adjusted for its position with a micrometer, while seeing fluorescence from the sample by the naked eye. Next, the sample is replaced by a diluted sample to be measured, and measurement is continued. Because Avalanshe photodiode has a small light-acceptance surface with a diameter of 0.2mm, it is impossible to place the optical system at a precise position while seeing by the naked eye. Accordingly, after positioning substantially with a concentrated solution, a sample is replaced by a diluted solution to be measured, laser-excited light is irradiated, and then the position of Avalanshe photodiode is finely adjusted using a micrometer such that the count of fluorescent photons per unit time becomes maximal.

Upon preparation of a sample, glass surface must be washed. Particularly when

dye molecules are dissolved in water, insufficient washing would cause adsorption of a dye on the glass surface. Normally, we degreased a glass surface with acetone, and treated it with 1M KOH then a 2:1 solution of 30%H_2O_2 and conc. H_2SO_4. As the result of such treatment, lipophilic components can be removed out of the glass surface, OH groups are regenerated on the surface, making the surface hydrophilic.

When water or an alcohol is used as a solvent, Raman scattering becomes a large background noise due to OH stretching vibration. For example, when rhodamine 6G fluorescence (maximum fluorescent wavelength 556nm) is measured with excited light at 488nm, Raman scattering appears at around 575nm and its overlap with fluorescent signals becomes large. To observe fluorescence which is sufficiently stronger than stray light or Raman scattering, the adjustment of the wavelength and intensity of excited light, as well as appropriate selection of a band pass filter, is required. Regarding the wavelength of excited light, when the wavelength of excited light is adjusted to the maximum absorption wavelength of a dye molecule (530 nm), the wavelengths of excitation and fluorescence are so close to one another that it becomes difficult to separate fluorescent signals from stray light. In contrast, the shorter the excitation wavelength, the better the separation of fluorescent signals from stray light. However, there are difficulties; that is, fluorescent signals become weaker because the absorption coefficient of a dye decreases at this excitation wavelength, and Raman scattering overlaps with fluorescent signals. It is thought that there also exists an optimum intensity in excited light. As the intensity increases, both stray light and Raman scattering become larger and, as a result, quenching of a fluorescent dye starts to occur. On the other hand, when the intensity is low, it takes a certain period of time until re-excitation ocuurs after dye molecules return to their ground state, so fluorescent signals become weaker. In a system as illustrated in Fig. 2, the best results can be obtained with an excitation wavelength of 514nm and an intensity of 0.5mW. Regarding a band-pass filter, an optimum penetration wavelength and its half width should be determined. Background noises, such as Raman scattering and stray light,

are minimized and the signal intensity is kept at a level which is not too low. We used a band-pass filter BP545-580 (Omega Optics, band width: 545nm-580nm).

Figure 2a shows the time-dependency of fluorescence when the gate time of a multichannel counter is set at 1.34ms. A single molecule can be detected when a rhodamine 6G solution is at a concentration of 10^{-10}M or less. Observed here is that rhodamine 6G molecules (in Brownian movement), which have entered into an observation area, repeat absorption of excited light and release of fluorescence at a life span of approximately 10ns, and release about 10^8 photons per sec (bunching). When these molecules leave the observation area, fluorescent signals quench (anti-bunching). Furthermore, the residence time of rhodamine 6G due to Brownian movement within the observation area is thought to be about 1ms. During the residence time, a single dye molecule releases about 10^6 photons. Since about 1,000 photons are measured in this case, the detection system detects photons in about 1% efficiency. Figure 2b shows the results when the gate time of the multichannel counter was changed to 80ns. This figure shows that a single dye molecule randomly repeated absorption of excited light and release of fluorescence at an average period of about 10ns.

Fig. 1 Diagram of a confocal laser fluorescent microscope system

Fig. 2 Time-dependency of fluorescent signal in a solution of water and ethanol (1:1) containing 10^{-10}M rhodamine 6G.

(a) Gate time of a multichannel counter was set at 1.34ms; the number of data points on the time axis was set to 1k. ○ denotes a bunching area; ◇ denotes an anti-bunching area. (b) Gate time of a multichannel counter was set at 80ns; the number of data points on the time axis was set to 16k. △ denotes time when one dye molecule enters into the observation area.